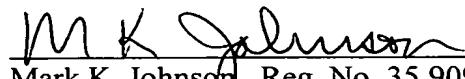


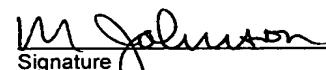
A computer readable form has also been submitted and it is the same as the paper copy that has been added. Additionally, the Specification has been amended according to the attached Specification replacement sheets. The sheets do not include new matter.

Respectfully submitted,



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plants and microorganisms) that include infectious disease, auto-immune disorders and disorders with an autoimmune component (such as rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis, ankylosing spondylitis, psoriasis, Reiter's Syndrome, fibromyalgia, dermatomyositis, polymyositis, scleroderma, 5 diabetes mellitus, glomerulonephritis), and cancer. These PSNA's could also be used to treat human and animal disorders that include cancer, infectious disease, auto-immune disorders and aspects of other disorders.

Brief Description of the Drawing

10

Figure 1. The percentage of phage recovered (phage recovered/phage injected X 100, mean of 2 experiments) of various T7 phage clones from the blood and liver 5 min after tail vein injections in mice *in vivo*. Our laboratory names for the T7 phage corresponding to their C-terminus sequences are shown below. T7 vector refers to the 15 vector coding sequence without any library inserts.

Methods: The phages (109 pfu/0.3ml PBS) were injected into tail vein of ICR male mice (5-6 weeks old) pre-treated (20-24h) with GdCl₃ at a dose of 10mg/kg. Three min after injection, heparin (20 unit/head) was injected by same route. Under anesthesia, blood was collected directly from heart and livers were perfused with 1 20 unit heparin/ml containing PBS 30 ml for 3 min, and then collected. Livers were homogenated with 3-fold of their weight of 2% Triton X-100/1M NaCl in PBS to lyse the cells and disperse the phage. The titration of phages was assessed by using *E. coli* BL21 in an appropriate dilution.

20/6 FQ*
25 32/77 FQS*
32/23 FSQV*
#112 FQSGVMLGDPN* (SEQ ID NO: 1)
#114 .. FQSGVMLGDPNSDGALRQSGRGKSSRP* (SEQ ID NO: 2)
T7 Vector FQSGVMLGDPNSSVDLAAALE* (SEQ ID NO: 3)

30

Detailed Description

The invention is described in the following sections: I) Methods of selection for phage that are less prone to inactivation, II) Peptide-display libraries and production of peptide-display phage libraries that are less prone to inactivation, III) 5 Phage that are less prone to inactivation for treating bacterial infections, IV) Methods for selection of serum proteins that bind specific peptide ligands, V) Peptides for drug and nucleic acid delivery, infections, VI) Methods for the production and uses of peptide-specific natural antibodies (PSNA).

10 **I. Methods of selection for phage that are less prone to inactivation**

We have discovered that bacteriophage (abbreviated as "phage") are inactivated in blood *in vivo* (e.g., in the systemic circulation of an animal) and in serum *in vitro* (in a test tube outside of the body) by natural antibodies and complement. The test tube indicates any type of container for holding a liquid and can 15 be made of glass or plastic. Inactivation means the loss of the phage's ability to infect bacteria. Phage infection can be assessed based on the ability of phage to lyse bacterial lawns. Therefore, the amount of phage is expressed in plaque forming units that correspond to the number of plaques (small clear areas) in a lawn of bacteria grown on an agar plate.

20 The phage can be inactivated by antibodies that activate complement upon binding to the phage. The antibodies can be natural antibodies that mostly belong to the IgM or IgG class. The phage can also be inactivated if it is biotinylated prior to exposure to the bodily fluid (e.g., blood *in vivo* or *in vitro*) and then allowed to interact with a probe that tightly binds biotin, such as avidin, streptavidin, neutravidin, or other protein derived from or related to these proteins.

In one embodiment of the invention, this inactivation process is used to select 25 for phage that are resistant to the inactivation process. The initial phage for this selection process can be (but not limited to) peptide display phage libraries that are derived from filamentous phage such as M13 or non-filamentous phage such as T7 phage. The initial phage can also be UV-irradiated to increase the number of mutations or derived from recombinant DNA that has been subjected to regular

polymerase chain reaction (PCR), error-prone PCR, or DNA shuffling or method to increase the variability or number of mutations in the phage sequence.

For the in vitro testing, blood or a blood derived material such as plasma, serum, or purified proteins and factors can be used in the inactivation process. For the 5 in vitro testing, serum derived from blood (by clotting) or citrated plasma (by adding Ca) can be used in the inactivation process. The complement-grade serum from commercial sources that is pre-filtered and lyophilized or snap-frozen can also be used. Prior to use, the serum pH can be left as is or adjusted to 7.2-7.6. In case of commercial serum, it is also filtered to remove protein precipitate formed during 10 freezing or lyophilization. With the serum depleted of particular proteins, purified proteins such as immunoglobulins (Ig) as a whole or Ig fractions that are enriched for IgG, IgM, IgA, or IgD can be used to reconstitute the ability of serum to inactivate phage. Purified factors that may be required for phage inactivation or protection by incomplete serum also include complement proteins, blood fractions containing 15 proteins greater than 25 kDa (kilodaltons) 50 kDa, 100 kDa, 200 kDa or 300 kDa in size. Blood, serum, plasma, or other blood-derived fractions can be subjected to purification processes that include precipitation, extraction, column chromatography methods and electrophoresis. Column chromatography methods include the following types of chromatography: size exclusion, ion-exchange, reverse phase, affinity 20 purification or any combination of these types. Electrophoresis includes the types of separation based on charge, pI, the change of protein mobility in the presence of particular ligands or any combination of these techniques.

For in vivo testing in the whole animal, the phage can be injected 25 intravenously or intraarterially into the systemic or pulmonary circulation. It can also be injected into blood vessels that supply the liver (hepatic artery, portal vein, hepatic vein, via the vena cava, via the aorta), kidneys, muscle (femoral, iliac, brachial, axilla arteries) or brain. The phage can also be injected into other body spaces that include the peritoneum (intraperitoneally) and cerebral spinal fluid (ventricular spaces, subdural, epidural). The animal can be a vertebrate such as fish, amphibians, reptiles or 30 mammals. Mammals include rodents (mice, rats), guinea pigs, hamsters, dogs, pigs, non-human primates (such as Rhesus) and humans. The animals can be particular

strains with certain features. These can be animals that are defective in the immune system and do not produce certain antibodies, for instance, IgM-deficient mice. These animals can also have defects in the complement system, such as the absence or functional impairment of particular complement proteins caused by natural or artificial deletions or mutations in the corresponding genes.

- 5 Phage that are resistant to inactivation can be obtained from a variety of tissues in vivo, including the blood, liver, lung, brain, muscle, spleen, kidneys, intestines, prostate, thymus, adrenal glands, thyroid, gonads, eyes, and skin. The resistance to inactivation results from association of phage with particular plasma 10 proteins that protect phage against inactivation by the complement system. Different plasma proteins bind to phage through specific recognition of different peptides or proteins or protein domains displayed at the phage surface. Such complexes of phage with plasma proteins can either stay in circulation or be targeted to particular locations through the interaction of bound plasma proteins with cell receptors. The 15 binding of bound plasma proteins to cell receptors is promoted by changes in the conformation of bound proteins and by a high level of cooperation on the protein-receptor interactions due to a high density of bound proteins on the phage surface. Therefore, the phage binds to particular organs through indirect targeting. Alternatively, if the peptides are exposed on the phage in the format that is not 20 recognized by natural antibodies , the phage can be used to select for the peptides that bind to cell receptors directly.

In one embodiment to measure the survival of phage T7 in blood, 10^9 pfu of phage per animal are injected into a tail vein and 100 μ l blood samples are collected from a non-injected tail vein (of the same animal) into 10 μ l (10U) of heparin on ice at 25 specified time points. Plasma is prepared by centrifugation. Phage from blood and plasma are detected by soft agar plating. Plating dilutions are done in LB medium (20 g of yeast extract, 40 g of tryptone and 20 g of NaCl per 1L of medium).

In a preferred embodiment, phage are subjected to more than one round of selection. Phage that are selected for resistance to inactivation are then grown on 30 bacteria to expand their number and then subjected to another inactivation process.

This can change the type of phage clones that are resistant to inactivation. It can also increase the percentage of phage that survive the inactivation process.

In other embodiments, factors that affect the inactivation process can be added to the test tube in vitro or to the living animal. These include small molecules or drugs such as phosphoryl choline and aminocaproic acid. Molecules that inhibit macrophage activity include gadolinium (GdCl_3), carrageenan and encapsulated bisphosphonates. Molecules that inhibit the complement system include antibodies, venoms (e.g cobra venom factor - CVF) and natural or artificial complement regulatory proteins represented by both membrane and soluble proteins. The function of the complement system in vitro can be also inhibited by heat (50°C for the alternative complement activation pathway and 56°C for the classical complement activation pathway) and chelators (EGTA or EDTA for the alternative complement activation pathway and EDTA or EGTA or EGTA/Mg for the classical complement activation pathway).

15 II. Peptide-Display Libraries and Production of Peptide-Display Phage Libraries That Are Less Prone to Inactivation

T7-based peptide display libraries are made by using Novagen T7 vectors. Both the vectors that give the phage with a high number of peptide copies per phage particle (displayed in all coat proteins) and the vectors that give just 0.1-10 copies of peptides of polypeptides per phage particles are used. Low-copy phage are grown either in the *E. coli* strains provided by Novagen (BLT5403 and BLT5615) or other strains. The bulk of the phage coat protein comes from the plasmid but the structure of this protein is different. The *E. coli* strains BLT5403 and BLT5615 produce T7 protein 10A. In other libraries, the bulk of the phage coat protein is a truncated 10B protein that shows a remarkable protection of phage against complement-mediated inactivation.

“Double” display libraries displaying: a) a “constant” peptide or protein that prevents serum inactivation (e.g. lys+/arg+ peptides) or is less prone to inactivation and b) a random peptide for selection of tissue, sub-cellular, or blood persistence properties.

For making “double” display libraries of T7 phage, bacteria such as E. coli are modified so that it expresses a T7 phage coat protein that is incorporated into phage that constitute a peptide-display library. It is double-display in that each phage has two different proteins in its coat; “protein A” that affects its interactions with blood or tissue and “protein B” that contains a “random” peptide sequence. This peptide sequence is “random” in that it is different among different phage clones that constitute a phage library. In one embodiment, protein A is selected for resistance to serum inactivation in vitro or prolonged blood circulation in vivo. In one embodiment protein A is derived from the phage clone 20-6 (Table 3) (AAGAVVFQ (SEQ ID NO: 4)) peptide sequence for coat protein carboxy-terminus).

Selected sequence:

MASMTGGQQMGTNQGKGVVAAGDKLALFLKVGGEVLTAARTSVTTSRH
MVRSISSGKSAQFPVLGRTQAAYLAPGENLDDKRKDIKHTEKVITIDGLLTAD
VLIYDIEDAMNHYDVRSEYTSQLGESLAMAADGAVLAEIAGLCNVESKYNEN
IEGLGTATVIETTQNKAALTDQVALGKEIIAALTKARAALTKNYVPAADRVFY
CDPDSYSAILAALMPNAANYAALIDPEKGSIRNVMGFEVVEVPHLTAGGAGT
AREGTTGQKHVFANKGEGNVKVAKDNIGLFMHRSAVGTVKLRDLALERA
RRANFQADQIIAKYAMGHGGLRPEAAGAVVFQ (SEQ ID NO: 5)

In other embodiments, protein A is derived from the phage clone 32-77 (AAGAVVFQS (SEQ ID NO: 6)) peptide sequence for coat protein carboxy-terminus) or phage clone 32-23 (AAGAVVFSQV (SEQ ID NO: 7)) peptide sequence for coat protein carboxy-terminus) (Table 3). In yet other embodiments, protein A is derived from phage clones listed in Table 1 and have a terminal lysine (Table 1A) or arginine (Table 1B) or contain a tyrosine (Table 1E). The gene encoding these different “protein A” are placed within a plasmid that is carried by a bacteria such as E. coli.

“Protein B” contains a “random” peptide sequence. This peptide sequence is “random” in that it is different among different phage clones that constitute a phage library. The random peptide sequence can be at the amino terminus, carboxy terminus or within the coat protein. The random peptide sequence can encode a peptide that is one to 25 amino acid residues in length. The random peptide can contain invariant

parts in addition to the random part. In one embodiment, the invariant part is derived from phage clones listed in Table 1 and has a terminal lysine (Table 1A) or arginine (Table 1B) or contains a tyrosine (Table 1E).

T7 phage libraries constructed with proteins "A" and "B" can have varying proportions of these proteins. In one embodiment, protein A constitutes 0.1, 1, 10, 20, or 50 percent of the coat proteins in the phage. Proteins A and B can contain two cys residues so that disulfide bonds are formed and peptide sequences are constrained.

In one embodiment, T7 libraries displaying random peptides within the 10B capsid protein are constructed using a random one- to 25-mer peptide insert by using simple second strand synthesis (O'Neil, et al., Methods in Enzymology, 245:370-86. 1994) and placed into the Eco RI/Hind III sites of the T7Select 415-1 vector arms (Novagen Corp., Madison, WI). The single-stranded sequence is an oligonucleotide (xxxGAATTGggacggtgcc SEQ ID NO: 123 (NNG/T)1-25
ggggctggaAAGCTTxxxxxx SEQ ID NO: 124). A 21-mer reverse primer (xxxxxxAAGCTTtccagcccc SEQ ID NO: 125) is used to fill in the complementary strand with exo⁻ Klenow fragment. Specific methods for cloning, propagation and maintenance are used as specified in the manual supplied with the T7Select Kit (Novagen). The complexity of our libraries generated by growing phage in the BL21 E. coli strain is determined.

In order to be able to draw on long peptides, along with short ones, two other E. coli host strains, BLT5615 and BLT5403 in addition to BL21m can be used. Both strains provide additional phage T7 coat protein, 10A, from a plasmid. According to our observations, the ratio of 10A and 10B proteins in the phage based on the vector T7Select415-1 is 4 to 1. A lower density of long peptides on the phage surface promotes phage survival in a mixed population. Decreasing the density of displayed peptides should be also useful while trying to select for high-affinity peptide ligands. It enables one to determine the number of peptides required for a certain effect.

Constrained phage T7 display containing X₂CX₃₋₂₀CX₂ peptides can also be used. It may be necessary to expose the phage to gentle oxidizing agents to form the disulfide bonds. It has been found that constrained peptide display libraries may be

more apt for finding a specific ligand but this may not be necessary for large peptides that can form secondary structure.

III. Phage for treating bacterial infections

5 Peptide-display phage that are less prone to inactivation can be used to treat bacterial infections. In one embodiment, a phage library is selected for clones that are resistant to serum inactivation in vitro and the clones resistant to serum inactivation are injected into an animal with a bacterial infectious disorder. The phage infects the bacteria and kills the bacteria, thus alleviating the infectious disease state in the
10 animal. The phage can be injected intravenously or into the tissue that is infected such as sinuses, pulmonary, prostate, gastrointestinal, or central nervous system (ventricular fluid, brain parenchyma, spinal cord). In one embodiment, the phage is a T7 phage. In another embodiment, the T7 phage is the phage clone 20-6 (Table 3) (AAGAVVFQ (SEQ ID NO: 8) peptide sequence for coat protein carboxy-terminus).

15

IV. Selection of serum proteins that bind specific peptide ligands

We have discovered that phage are rapidly inactivated by blood, serum and other blood derivatives and factors in vitro and in the circulating blood in vivo. Phage clones can be selected that are resistant to this inactivation process. In some instances, 20 the phage clones that are resistant to inactivation bind to blood constituents such as serum proteins. In a preferred embodiment a blood constituent binds to the displayed peptide sequence that is unique or specific to that particular phage clone. (That is, each phage clone displays a specific peptide sequence that is related to a DNA sequence and that is specific to the specific phage clone. Depending on the complexity of the phage library the phage clone and thus the peptide sequence may or 25 may not be unique.)

The blood constituent that binds to the peptide (cognate to the phage clone that is resistant to inactivation) can be purified and identified on the basis of the affinity of the blood constituent for the peptide. In one embodiment, affinity purification can be 30 accomplished by affinity chromatography.

Yet in another embodiment, affinity purification can be done by affinity centrifugation. In another embodiment, affinity purification can be performed using affinity precipitation. For example, antibodies against the phage can be used to immunoprecipitate the phage with the blood constituent attached to the phage.

- 5 In another embodiment, affinity purification can be brought about by filtering. In one instance, filters that do not let the phage pass through filters can be used to purify the phage and the blood constituent attached to it away from the rest of the blood constituents that do not bind the phage as tightly.

In all these types of affinity purification, the phage can be washed to enrich for
10 a blood constituent that binds the phage clone with different affinities or via different methods. The washes can contain various concentrations of salt. The washes can also contain detergents. The washes can also contain specific ligands such as phosphatidyl choline, free peptides, and peptides attached to proteins and other supports.

In a preferred embodiment, after the blood constituent and the phage are
15 purified from the rest of the blood, the blood constituent bound to the phage is separated from the phage and the blood constituent is identified using techniques such as protein gel electrophoresis, two-dimensional electrophoresis, immunoblotting, and protein sequencing.

20 **V. Peptides for biologically-active compound and nucleic acid delivery**

Phage clones that are resistant to inactivation can be used to derive peptides that are of use for drug and nucleic acid delivery. In one embodiment, they are peptides that have a terminal lysine or arginine at the carboxy terminus. In another embodiment, they are peptides that contain a tyrosine. In yet another embodiment, 25 they are peptides that have a terminal lysine or arginine at the carboxy terminus or they are peptides that contain a tyrosine and that are used to prolong the circulation life or persistence of the biologically-active compound or nucleic acid delivery particle in the circulation or render the drug or nucleic acid delivery particle more resistant to inactivation.

30 In still another embodiment, the peptide contains a cell targeting signal and a terminal lysine or arginine at the carboxy terminus. The peptide can also contain a cell

targeting signal and a tyrosine. The peptides that contain a cell targeting signal and a terminal lysine or arginine at the carboxy terminus and that are used to prolong the circulation life or persistence of the biologically-active compound or nucleic acid delivery particle in the circulation or render the drug or nucleic acid delivery particle more resistant to inactivation.

5 A biologically-active compound or nucleic acid delivery particle consists of a biologically-active compound or nucleic acid and amphipathic compounds. In another embodiment, the drug or nucleic acid delivery particle can consist of a drug or nucleic acid and liposomes. Also, the drug or nucleic acid delivery particle can consist of a
10 drug or nucleic acid and a polymer. The polymer can be a polyion such as a polycation or polyanion. Yet in another embodiment, the drug or nucleic acid delivery particle can consist of a drug or nucleic acid, an amphipathic compound, and a polymer. Still, in another embodiment, the peptide could have the carboxy terminus blocked so that there is not unmodified carboxy group at the "carboxy" terminus or
15 the peptide could have the amino terminus blocked so that there is no unmodified amino group at the "amino" terminus.

In yet another embodiment, random peptides or the peptides with the primary structure corresponding to the primary structure of the proteins from the recipient species are used to target nucleic acids or drug carriers to the liver. Also, the delivery
20 particles derivatized with random peptides or the peptides with the structure derived from the proteins of the recipient species are used to decrease immunogenicity of conjugated peptides.

VI. Peptide-specific natural antibodies

25 Peptide-specific natural antibodies (PSNA) are natural antibodies that reacts specifically with a peptide in some demonstrable way such as inducing inactivation of phage that display multiple peptides. It can also be demonstrated by enzyme linked immunoassay (ELISA) or radioimmunoassay (RIA) or by affinity chromatography.

In one embodiment, the PSNA's are IgM or IgG.
30 In another embodiment, PSNA's are purified from serum or plasma by using synthetic peptides conjugated to the resin. The PSNA's bound to the peptides on the

column can be eluted by low pH or high pH buffers, by the buffers containing chaotropic agents or high salt or by the buffers that combine any of these properties. The PSNA's with low affinity can be isolated by isocratic elution of the column with the same buffer that was used to apply serum or plasma on to the column.

5 In another embodiment, PSNA's are produced using recombinant methods- include phage as well. V_H and V_L immunoglobulin genes are isolated from a pool of "naive" B-cells and used to construct single-chain antibodies exposed either on M13 or T7 phage. The phage is panned against UV-inactivated, immobilized T7 phage displaying specific peptides or a whole library. Alternatively, the phage is panned
10 against immobilized synthetic peptides. The bound live phage is collected and amplified. The selection process is repeated and selected immunoglobulins characterized in terms of their affinity toward target peptides and the ability to activate C if converted into a physiological form recognized by C1q.

In yet another embodiment, PSNA's can be produced using monoclonal
15 methods. B-cells producing PSNA's are selected by using fluorescently labeled phage displaying peptides of interest. The selected B-cells are immortalized using conventional techniques, cloned and the antibodies secreted by resultant clones are characterized for their ability to bind peptides of interest and activate C once bound.

In another embodiment, PSNA-producing B-cells are selected by using
20 antiidiotypic antibodies that specifically bind to PSNA's.

PSNA's can be used for diagnostic or detection purposes. These include detecting the presence of a particular peptide sequence in a protein. The protein containing the specific peptide sequence or a peptide can be in a mixture such as in an extract from a tissue or a tissue section. The protein or peptide can be on a membrane, glass, or plastic structure. The protein or tissue section could be first digested with an enzyme and then probed with the PSNA. Binding of the PSNA to the peptide sequence can be detected by a variety of methods. These include the attachment of a reporter or marker molecule directly to the PSNA or indirectly. In one embodiment, another antibody or secondary antibody containing a reporter or marker molecule is
30 used to detect the presence of the PSNA. The PSNA can be used in immunoblots,

ELISA, RIA, immunohistochemical assays, fluorescence polarization, or Biocore-type binding assays.

PSNA's can also be used for selective visualization or labeling of particular tissues or cells. PSNA's can be indirectly selected for this purpose from a natural pool
5 of PSNA's or from B-cell libraries by using the phage that displays a repertoire of peptides characteristic of certain cells or tissues. Such phage can be selected by pre-incubating serum with the cells or tissues of interest followed by collecting the phage that survives in the serum pre-incubated with the target tissue. Prior to these steps, the target cells can be pre-treated with proteases that would generate exposed protein
10 carboxy-termini or permeabilized with detergents or fixed and then permeabilized with detergents. The treatment of cells with detergent is used to expose protein carboxy-termini on the inner side of the cell plasma membrane and on the cell organells. A combination of proteolysis and permeabilization can also be used. The pre-existing and/or protease-generated repertoire of carboxy-termini on the plasma
15 membrane and cell organelles can be different for different types of cells. Therefore, different PSNA's would be removed from the serum pre-exposed to different cells . The phage displaying corresponding peptides would survive in the absence of cell- or tissue-specific PSNA's and could be used after a few rounds of selection as a probe for the isolation of B-cells producing PSNA's against these peptides. Corresponding
20 synthetic peptides could be used for affinity purification of pre-existing PSNA's that would selectively react with different cells and tissues. The staining of cells or tissues with selected PSNA's can be done using conventional secondary antibodies or by using the antibodies against C proteins. In the latter case, the complement deposition will be specifically induced by the multivalent binding of PSNA's to closely spaced
25 protein carboxy-termini. Spurious binding events will not generate complement activation and, therefore, will not be detected.

A similar approach can be used to generate PSNA's that would stain particular protein bands or spots immobilized on paper, plastic or glass supports. The immobilized proteins could be incubated with serum as is or after mild treatment with
30 proteases. The serum depleted of particular PSNA's would be used to select phage with corresponding peptides.

The different individual types of PSNA's can be correlated with the prevalence, incidence, penetration, or likelihood of an individual having a specific disease or disorder. This would be akin to correlating a particular genotype to a phenotype. In fact, the genetic loci for encoding the PSNA's could be used for this purpose as well.

PSNA's can also be used to treat disease in living creatures and organisms such as animals and humans. The disease can be, but not limited to, an infectious disease, cancer, autoimmune disorder, inflammatory condition, cardiovascular disorder, or nervous disorder. The PSNA can be injected into the blood or a tissue. It can be a part of the PSNA such as the part that binds the peptide. The PSNA that is used for therapeutic purposes can be purified from a bodily fluid, produced by recombinant methods, or by "monoclonal" methods. The PSNA can be "humanized" in that parts of the PSNA that are non-essential for binding to the peptide are removed and replaced with parts of human antibodies. The PSNA can also be linked to another biologically-active compound or protein such as a toxin (e.g., diphtheria or pertussis)

Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

20 **Biologically active compound**

A biologically-active compound is a compound having the potential to react with biological components. More particularly, biologically active compounds utilized in this specification are designed to change the natural processes associated with a living cell. For purposes of this specification, a cellular natural process is a process that is associated with a cell before delivery of a biologically active compound. In this specification, the cellular production of, or inhibition of a material, such as a protein, caused by a human assisting a molecule to an in vivo cell is an example of a delivered biologically active compound. Pharmaceuticals, proteins, peptides, polypeptides, hormones, cytokines, antigens, viruses, oligonucleotides, enzymes and nucleic acids are examples of biologically active compounds.

Peptide and polypeptide refer to a series of amino acid residues, more than two, connected to one another by amide bonds between the alpha-amino group and carboxyl group of contiguous amino acid residues. The amino acids may be naturally occurring or synthetic. Polypeptide includes proteins and peptides, modified proteins and peptides, and non-natural proteins and peptides. Enzymes are proteins evolved by the cells of living organisms for the specific function of catalyzing chemical reactions. A chemical reaction is defined as the formation or cleavage of covalent or ionic bonds. Bioactive compounds may be used interchangeably with biologically active compound for purposes of this application.

10

Delivery of Biologically-Active Compound

The delivery of a biologically-active compound is commonly known as "drug delivery". "Delivered" means that the biologically-active compound becomes associated with the cell or organism. The compound can be in the circulatory system, intravessel, extracellular, on the membrane of the cell or inside the cytoplasm, nucleus, or other organelle of the cell.

Parenteral routes of administration include intravascular (intravenous, intraarterial), intramuscular, intraparenchymal, intradermal, subdermal, subcutaneous, intratumor, intraperitoneal, intrathecal, subdural, epidural, and intralymphatic injections that use a syringe and a needle or catheter. An intravascular route of administration enables a polymer or polynucleotide to be delivered to cells more evenly distributed and more efficiently expressed than direct injections. Intravascular herein means within a tubular structure called a vessel that is connected to a tissue or organ within the body. Within the cavity of the tubular structure, a bodily fluid flows to or from the body part. Examples of bodily fluid include blood, cerebrospinal fluid (CSF), lymphatic fluid, or bile. Examples of vessels include arteries, arterioles, capillaries, venules, sinusoids, veins, lymphatics, and bile ducts. The intravascular route includes delivery through the blood vessels such as an artery or a vein. An administration route involving the mucosal membranes is meant to include nasal, bronchial, inhalation into the lungs, or via the eyes. Other routes of administration include intraparenchymal into tissues such as muscle (intramuscular), liver, brain, and

kidney. Transdermal routes of administration have been effected by patches and iontophoresis. Other epithelial routes include oral, nasal, respiratory, and vaginal routes of administration.

5 **Nucleic Acid**

The term "nucleic acid" is a term of art that refers to a polymer containing at least two nucleotides. "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include
10 natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and synthetic derivatives of purines and pyrimidines, or natural analogs. Nucleotides are the monomeric units of nucleic acid polymers. A "polynucleotide" is distinguished here from an "oligonucleotide" by containing more than 80 monomeric units; oligonucleotides contain from 2 to 80 nucleotides. The term nuclei acid includes
15 deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The term encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudouracil, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil,
20 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine,
25 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and
30 2,6-diaminopurine.

DNA may be in the form of anti-sense, plasmid DNA, parts of a plasmid DNA, product of a polymerase chain reaction (PCR), vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives of these groups. RNA may be in the form of oligonucleotide 5 RNA, tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, ribozymes, chimeric sequences, or derivatives of these groups.

"Anti-sense" is a polynucleotide that interferes with the function of DNA and/or RNA. This may result in suppression of expression. Natural nucleic acids 10 have a phosphate backbone, artificial nucleic acids may contain other types of backbones and bases. These include PNAs (peptide nucleic acids), phosphothionates, and other variants of the phosphate backbone of native nucleic acids. In addition, DNA and RNA may be single, double, triple, or quadruple stranded.

The term "recombinant DNA molecule" as used herein refers to a DNA 15 molecule that is comprised of segments of DNA joined together by means of molecular biological techniques."Expression cassette" refers to a natural or recombinantly produced polynucleotide molecule that is capable of expressing protein(s). A DNA expression cassette typically includes a promoter (allowing transcription initiation), and a sequence encoding one or more proteins. Optionally, 20 the expression cassette may include transcriptional enhancers, non-coding sequences, splicing signals, transcription termination signals, and polyadenylation signals. An RNA expression cassette typically includes a translation initiation codon (allowing translation initiation), and a sequence encoding one or more proteins. Optionally, the expression cassette may include translation termination signals, a polyadenosine 25 sequence, internal ribosome entry sites (IRES), and non-coding sequences.

A nucleic acid can be used to modify the genomic or extrachromosomal DNA sequences. This can be achieved by delivering a nucleic acid that is expressed. Alternatively, the nucleic acid can effect a change in the DNA or RNA sequence of the target cell. This can be achieved by homologous recombination, gene conversion, 30 or other yet to be described mechanisms.

Gene

The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor (*e.g.*, - myosin heavy chain). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (*e.g.*, enzymatic activity, ligand binding, signal transduction, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene and the including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences that are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

As used herein, the terms "an oligonucleotide having a nucleotide sequence encoding a gene" and "polynucleotide having a nucleotide sequence encoding a gene," means a nucleic acid sequence comprising the coding region of a gene or in other words the nucleic acid sequence which encodes a gene product. The coding region

may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide or polynucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as

enhancers/promoters, splice junctions, polyadenylation signals, *etc.* may be placed in

- 5 close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript.

Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, *etc.* or a combination of both endogenous and

- 10 exogenous control elements.

The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is such present in a

- 15 form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids as nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a

- 20 mixture with numerous other mRNAs that encode a multitude of proteins. However, isolated nucleic acid encoding a given protein includes, by way of example, such nucleic acid in cells ordinarily expressing the given protein where the nucleic acid is

in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic

- 25 acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand (*i.e.*, the oligonucleotide or polynucleotide may be single-stranded), but may contain both the sense and anti-sense strands (*i.e.*, the

- 30 oligonucleotide or polynucleotide may be double-stranded).

Gene Expression

As used herein, the term "gene expression" refers to the process of converting genetic information encoded in a gene into RNA (*e.g.*, mRNA, rRNA, tRNA, or snRNA) through "transcription" of the gene (*i.e.*, via the enzymatic action of an RNA polymerase), and for protein encoding genes, into protein through "translation" of mRNA. Gene expression can be regulated at many stages in the process. "Up-regulation" or "activation" refers to regulation that increases the production of gene expression products (*i.e.*, RNA or protein), while "down-regulation" or "repression" refers to regulation that decrease production. Molecules (*e.g.*, transcription factors) that are involved in up-regulation or down-regulation are often called "activators" and "repressors," respectively.

Delivery of Nucleic Acids

The process of delivering a polynucleotide to a cell has been commonly termed "transfection" or the process of "transfecting" and also it has been termed "transformation". The polynucleotide could be used to produce a change in a cell that can be therapeutic. The delivery of polynucleotides or genetic material for therapeutic and research purposes is commonly called "gene therapy". The delivery of nucleic acid can lead to modification of the DNA sequence of the target cell.

The polynucleotides or genetic material being delivered are generally mixed with transfection reagents prior to delivery. The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into

the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells which have taken up

- 5 foreign DNA but have failed to integrate this DNA. The term "naked polynucleotides" indicates that the polynucleotides are not associated with a transfection reagent or other delivery vehicle that is required for the polynucleotide to be delivered to a cell.

A "transfection reagent" or "delivery vehicle" is a compound or compounds that bind(s) to or complex(es) with oligonucleotides, polynucleotides, or other desired

- 10 compounds and mediates their entry into cells. Examples of transfection reagents include, but are not limited to, cationic liposomes and lipids, polyamines, calcium phosphate precipitates, histone proteins, polyethylenimine, and polylysine complexes (polyethylenimine and polylysine are both toxic). Typically, when used for the delivery of nucleic acids, the transfection reagent has a net positive charge that binds
15 to the polynucleotide's negative charge. For example, cationic liposomes or polylysine complexes have net positive charges that enable them to bind to DNA or RNA.

20 **Polymer**

A polymer is a molecule built up by repetitive bonding together of smaller units called monomers. A polymer is defined as a compound containing more than two monomers. A monomer is a compound that can be attached to itself or another monomer and thus form a polymer.

- 25 In this application, the term polymer includes both oligomers which have two to about 80 monomers and polymers having more than 80 monomers. The polymer can be linear, branched network, star, comb, or ladder types of polymer. The polymer can be a homopolymer in which a single monomer is used or can be copolymer in which two or more monomers are used. Types of copolymers include alternating,
30 random, block and graft.

Polyion

A polycation is a polymer containing a net positive charge, for example poly-L-lysine hydrobromide. The polycation can contain monomer units that are charge positive, charge neutral, or charge negative, however, the net charge of the polymer must be positive. A polycation also can mean a non-polymeric molecule that contains two or more positive charges. A polyanion is a polymer containing a net negative charge. The polyanion can contain monomer units that are charge negative, charge neutral, or charge positive, however, the net charge on the polymer must be negative. A polyanion can also mean a non-polymeric molecule that contains two or more negative charges. The term polyion includes polycation, polyanion, zwitterionic polymers, and neutral polymers. The term zwitterionic refers to the product (salt) of the reaction between an acidic group and a basic group that are part of the same molecule.

Cell Targeting Signals

Cell targeting signal (or abbreviated as the Signal) is defined in this specification as a molecule that modifies a biologically active compounds such as drug or nucleic acid and can direct it to a cell location (such as tissue) or location in a cell (such as the nucleus) either in culture or in a whole organism. By modifying the cellular or tissue location of the foreign gene, the function of the biologically-active compound can be enhanced.

The cell targeting signal can be a protein, peptide, lipid, steroid, sugar, carbohydrate, (non-expresssing) polynucleic acid or synthetic compound. The cell targeting signal enhances cellular binding to receptors, cytoplasmic transport to the nucleus and nuclear entry or release from endosomes or other intracellular vesicles.

The cell targeting signal can be a ligand that binds to its cognate receptor.

Nuclear localizing signals enhance the targeting of the pharmaceutical into proximity of the nucleus and/or its entry into the nucleus. Such nuclear transport signals can be a protein or a peptide such as the SV40 large T ag NLS or the nucleoplasmin NLS. These nuclear localizing signals interact with a variety of nuclear transport factors such as the NLS receptor (karyopherin alpha) which then interacts with karyopherin beta. The nuclear transport proteins themselves could also function

as NLS's since they are targeted to the nuclear pore and nucleus. For example, karyopherin beta itself could target the DNA to the nuclear pore complex. Several peptides have been derived from the SV40 T antigen. These include a short NLS (H-CGYGPKKKRKVGG-OH (SEQ ID NO: 9)) or long NLS's (H-

- 5 CKKKSSSDDEATADSQHSTPPKKRKVEDPKDFPSELLS-OH (SEQ ID NO: 10)
and H-CKKKWDDEATADSQHSTPPKKRKVEDPKDFPSELLS-OH (SEQ ID NO: 11). Other NLS peptides have been derived from M9 protein
(CYNDFGNYNNQSSNFGPMKQGNFGRSSGPY (SEQ ID NO: 12)), E1A (H-CKRGPKRPRP-OH (SEQ ID NO: 13)), nucleoplasmin (H-
10 CKKAVKRPAATKKAGQAKKKL-OH (SEQ ID NO: 14)), and c-myc (H-CKKGPAAKRVKLD-OH (SEQ ID NO: 15)).

Signals that enhance release from intracellular compartments (releasing signals) can cause DNA release from intracellular compartments such as endosomes (early and late), lysosomes, phagosomes, vesicle, endoplasmic reticulum, golgi apparatus, trans golgi network (TGN), and sarcoplasmic reticulum. Release includes movement out of an intracellular compartment into cytoplasm or into an organelle such as the nucleus. Releasing signals include chemicals such as chloroquine, bafilomycin or Brefeldin A1 and the ER-retaining signal (KDEL sequence), viral components such as influenza virus hemagglutinin subunit HA-2 peptides and other types of amphipathic peptides.

Cellular receptor signals are any signal that enhances the association of the biologically active compound with a cell. This can be accomplished by either increasing the binding of the compound to the cell surface and/or its association with an intracellular compartment, for example: ligands that enhance endocytosis by enhancing binding the cell surface. This includes agents that target to the asialoglycoprotein receptor by using asialoglycoproteins or galactose residues. Other proteins such as insulin, EGF, or transferrin can be used for targeting. Peptides that include the RGD sequence can be used to target many cells. Chemical groups that react with sulphhydryl or disulfide groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as lipids fatty acids,

cholesterol, dansyl compounds, and amphotericin derivatives. In addition viral proteins could be used to bind cells.

Amphipathic Compounds

- 5 Amphipathic compounds have both hydrophilic (water-soluble) and hydrophobic (water-insoluble) parts. Hydrophilic groups indicate in qualitative terms that the chemical moiety is water-preferring. Typically, such chemical groups are water soluble, and are hydrogen bond donors or acceptors with water. Examples of hydrophilic groups include compounds with the following chemical moieties;
- 10 carbohydrates, polyoxyethylene, peptides, oligonucleotides and groups containing amines, amides, alkoxy amides, carboxylic acids, sulfurs, or hydroxyls. Hydrophobic groups indicate in qualitative terms that the chemical moiety is water-avoiding. Typically, such chemical groups are not water soluble, and tend not to hydrogen bonds. Hydrocarbons are hydrophobic groups.

15

Peptides

Peptides are polymers of amino acid residues and their derivatives. The peptide contains 1 or more amino acids and can be synthesized by artificial synthetic methods or in a living organism. The amino acid residues are joined by peptide bonds.

- 20 A peptide bond is one which the carboxy group of one amino acid is united with the amino group of another amino acid, with elimination of a molecular of water, thus forming a peptide bond: -CO-NH-. The peptide can contain amino acid derivatives or analogs such as d-forms of amino acids or β-amino acids.

25 **Blood and Its Constituents**

According to Stedman's dictionary, blood is the "fluid and its suspended formed elements that circulated through the heart, arteries , capillaries, and veins. The constituents of the blood are the non-cellular parts: serum, plasma, and the cellular parts: red blood cells, white blood cells (leukocytes), and platelets. Plasma is the fluid (non-cellular) part of the blood of the blood that is distinguished from the serum obtained after coagulation. Serum is the fluid portion of the blood after removal of the

fibrin clot and blood cells, distinguished from the plasma in circulating blood. The non-cellular parts of blood also consist of complement and clotting factors. A blood product is a substance that is formed from blood or purified from blood.

5 Complement (C)

It is a thermolabile substance, normally present in serum, that is destructive to certain bacteria and other cells which have been sensitized by specific complement-fixing antibody. The C system comprises more than 30 plasma or membrane proteins. The activation of C relies on a cascade of proteolytic steps performed by the protease domains in the components involved. There are three distinct C activation pathways: the classical pathway triggered by target-bound antibody, the MB Lectin pathway triggered by polysaccharide structures of microbes, and the alternative pathway triggered by the recognition of exogenous structures by the C components themselves. For historical reasons, the components of the C system are numbered from C1 to C9, with the biochemical reaction sequence being C1-C4-C2-C3-C5-C6-C7-C8-C9.

C1 (a complex of three subunits, C1q, C1r, and C1s) after activation by antibody-antigen complex or other activators is enzymic (as C1 esterase) for C4 and (owing to the reaction with C4) for C2. The C42 moiety (C3 convertase) of the C142 complex then cleaves C3, the active fragment of which enters the C1423 complex that cleaves C5. The complex C14235 then combines sequentially with C6, C7, C8, and C9 to form lytic complement. C1 may be activated also by aggregated antibody. C3 may also be activated by bacterial endotoxin, by the properdin system, and by a component of cobra venom.

25 Antibodies

One or other classes of globulins (immunoglobulins) present in the blood serum or body fluids of an animal. The classes include IgG, IgM, IgA, IgD and IgE.

Natural Antibodies

Natural antibodies are antibodies whose appearance in the blood does not require immunization with the corresponding antigen. According to Stedman's Medical Dictionary (Williams and Wilkins Co., Baltimore- 23rd edition, 1976),

"Originally, antibody was a body or substance evoked in man or other animal by an antigen, and characterized by reacting specifically with the antigen in some demonstrable way- antibody and antigen each being defined in terms of the other, but it is now supposed that antibodies may also exist naturally without being present as a 5 result of the stimulus provided by the introduction of an antigen.

Phage

Phage is also known as bacteriophage and is a virus that infects bacteria or has an affinity for bacteria. They can contain either DNA or RNA. They may contain 10 either single- or double-stranded nucleic acid. They can have various shapes and sizes. They can use different bacterial strains or species as a host. They can lyze the infected cells or just use the cells for reproduction.

Reporter or Marker Molecules

15 Reporter or marker molecules are compounds that can be easily detected. Typically they are fluorescent compounds such as fluorescein, rhodamine, Texas red, cy 5, cy 3 or dansyl compounds. They can be molecules that can be detected by infrared, ultraviolet or visible spectroscopy or by antibody interactions or by electron spin resonance. Biotin is another reporter molecule that can be detected by labeled 20 avidin. Biotin could also be used to attach targeting groups.

EXPERIMENTAL SECTION

25

Example 1- Selection for Phage Persisting in Blood

We studied the interaction of displayed peptides with blood constituents using a T7 phage display library based on the Novagen T7Select415-1 vector. Each phage particle displayed 415 copies of a corresponding peptide at the carboxy-terminus of 30 all copies of the phage coat protein 10B (Novagen T7Select System Manual, 1996).

The phage with displayed peptides mimicked fairly well the delivery vehicles with the peptide ligands linked to the vehicles through the peptide amino-termini.

We found that the titer of the T7 phage peptide library in the plasma of rats preinjected with GdCl₃ (to inhibit macrophages (Mizgerd, et al., J. Leukoc. Biol., 59:189-95, 1996)) decreased by 95-99% within 5 min after phage injection. The rapid decrease in the phage titer was not accompanied by phage accumulation in blood cells, liver, kidneys, spleen, lungs, heart and skeletal muscles (data not shown). Therefore, the phage appeared to be functionally inactivated in blood. This was confirmed by observing phage inactivation in rat serum *in vitro*. Typical recovery of 10 phage after incubating phage library (10⁷ pfu) with 100 µl of serum at 37°C for 30 min was less than 1%.

In contrast to the phage library, wild-type T7 survived *in vivo* quite well. 15-20% of wild-type phage was recovered from plasma in 5 min after injection. Since the library phage and wild-type phage were different only in the structure of the carboxy-terminal part of their coat proteins (Novagen T7Select System Manual, 1996), this suggested that the inactivation of the library was associated, at least in part, with the structure of the protein 10B C-termini displaying peptides.

The mechanism of phage inactivation by blood constituents was studied using non-selected phage and the phage selected for persistence in the blood of rats over 60 min in an infectious state. The phage yield from the plasma of rats in the 1st round of selection was approximately 0.01%. The yield rose to approximately 15% in the 2nd round and stayed around this level through 3 following rounds of selection. After the 5th round, 33 individual clones were isolated and sequenced. All sequenced clones displayed peptides with either a K or an R residue at the carboxy-terminus (Table 1, A and B). These clones were hence designated K+ and R+ clones (K+/R+ as a class), respectively. Besides the carboxy-terminal K and R residues, there appeared to be preference for certain amino acid residues at other peptide positions (marked in bold in Table 1, A and B). Non-selected phage were randomly isolated from the initial library and divided into K+/R+ and K-/R-clones (Table 1, C and D).

Table 1. Primary structure of selected and non-selected T7 phage clones.

Clone	Peptide	Clone	Peptide
A. Selected K+ Phage		C. Non-Selected K+/R+ Phage	
19-4	QVTK (SEQ ID NO: 16)	IL-2	AVK (SEQ ID NO: 17)
19-5	AK	IL-7	QLVRVISR (SEQ ID NO: 18)
19-6	VVVESVPK (SEQ ID NO: 19)	IL-15	R
19-7	ARPVQK (SEQ ID NO: 20)	L5-3	NSR (SEQ ID NO: 21)
19-9	GRLK (SEQ ID NO: 22)	L5-7	RKSLR (SEQ ID NO: 43)
19-15	AFTNK (SEQ ID NO: 23)	L5-10	RK
19-16	VTPQVK (SEQ ID NO: 24)		
19-17	AVK		
19-18	DNTPKTK (SEQ ID NO: 25)	D. Non-Selected K-/R- Phage	
19-19	SLK	IL-1	IEFSG (SEQ ID NO: 44)
19-20	HRPKEGGKPALK (SEQ ID NO: 26)	IL-8	*
19-22	RTNPKVVK (SEQ ID NO: 27)	IL-9	MVLPFQQTVA (SEQ ID NO: 45)
19-24	TTRTPK (SEQ ID NO: 28)	IL-11	QSANI (SEQ ID NO: 46)
19-25	NNAQGARVK (SEQ ID NO: 29)	IL-14	KIPY (SEQ ID NO: 47)
19-28	MATVK (SEQ ID NO: 30)	IL-16	LPSGG (SEQ ID NO: 48)
19-29	KLRLMK (SEQ ID NO: 31)	IL-20	YNAKTDRG (SEQ ID NO: 49)
19-30	GVREPK (SEQ ID NO: 32)	IL-21	L
19-31	PTIK (SEQ ID NO: 33)	IL-23	KTNVEKGPM (SEQ ID NO: 50)
19-32	SRASVKGSTK (SEQ ID NO: 34)	IL-27	NSNAGLENH (SEQ ID NO: 51)
19-33	IK	IL-32	IQL
19-35	TK	L5-1	ME
19-37	KTK	L5-8	MVRRV (SEQ ID NO: 52)
19-38	RKPQK (SEQ ID NO: 35)	L5-9	LSARAP (SEQ ID NO: 53)
19-40	KVREK (SEQ ID NO: 36)		
B. Selected R+ Phage		E. Selected Y+ Phage	
19-1	GGR	32-2	RSYR (SEQ ID NO: 54)
19-3	ASRVR (SEQ ID NO: 37)	32-3	QESRTETDSQYLA (SEQ ID NO: 55)
19-10	RER	32-5	QGDYT (SEQ ID NO: 56)
19-11	KSGGPAER (SEQ ID NO: 38)	32-16	MQYS (SEQ ID NO: 57)
19-13	RRRNFER (SEQ ID NO: 39)	32-17	YRA
19-14	MDSMSNTPNGSER (SEQ ID NO: 40)	32-22	YGPQQ (SEQ ID NO: 58)
19-21	PSSQQAQR (SEQ ID NO: 41)	32-24	VDY
19-36	KNMR (SEQ ID NO: 42)	32-26	GKGKTDDPRYQKFT (SEQ ID NO: 59)
19-39	QR	32-28	AATGSDQGLNKAY (SEQ ID NO: 60)

Legend to Table 1: Bold letters that designate frequently occurring amino acid residues in K+/R+ (A and B) and Y residues in Y+ (F) peptides. "*" designates stop-codons.

Both selected and non-selected K+/R+ phage persisted in plasma in infectious 5 state much longer than K-/R- phage (Fig. 1A). None of the tested K-/R- clones, featuring altogether 10 different carboxy-terminal peptide residues (Table 1D), showed significant presence in plasma (Fig. 1A). Therefore, the persistence of K+/R+

phage in circulation was mainly due to the presence of a K or an R residue at the peptide carboxy-terminus. K+/R+ clones, in contrast to K-/R- clones, also showed a high level of survival in serum (Table 2, A, D and G). This suggested a common mechanism for K+/R+ phage protection *in vivo* and *in vitro*.

5

Table 2. The effect of various conditions on phage inactivation *in vitro*.

	Clone	Serum Treatment	Additions	% Phage Survival*
A.	K+	none	none	52.0 ± 19.6
B.	K+	Lysine-Sepharose	none	1.8 ± 2.1
C.	K+	Lysine-Sepharose	CRP	53.5 ± 19.8
D.	R+	none	none	44.5 ± 9.0
E.	R+	Lysine-Sepharose	none	0.4 ± 0.5
F.	R+	Lysine-Sepharose	CRP	37.0 ± 13.3
G.	K-/R-	none	none	0
I.	K-/R-	Lysine-Sepharose	none	0.3 ± 0.3
J.	K-/R-	Lysine-Sepharose	CRP	0.1 ± 0.3
K.	K+/R+	none	200 mM PC	0

Legend for Table 2: [* mean ± standard deviation. The effect of CRP on the survival of K+/R+ phage in Lysine-Sepharose-treated serum was assessed by incubating 10 µl (10⁶ pfu) of phage with 300 µl of serum supplemented with 10 µg of CRP. The total

10 volume of the samples was adjusted to 500 µl with PBS/0.68 mM CaCl₂. The samples were incubated at 37°C for 30 min, and examined for the presence of infectious phage by a plaque forming assay (Novagen, Novagen T7Select System Manual, 1996). The phage survival percentage was calculated using the data for 5 different clones with the same type of the protein 10B carboxy-terminus.]

15

METHODS:

T7 Phage Peptide Display Library.

The vector T7Select415-1 (NSSSVDKLAAALE SEQ ID NO: 121) (Novagen, Madison, WI) was employed to

20 display random peptides (NSDGA(X)₂₀GAVKLAAALE SEQ ID NO: 122) at the carboxy-terminus of all phage coat protein 10B molecules (Novagen T7Select System Manual, 1996). The expression of the second coat protein, 10A, was disabled (Novagen T7Select System Manual, 1996). The vector insert was generated from the

oligonucleotide xxxGAATT~~C~~ggacggtgcc SEQ ID NO: 123 (NNG/T)₂₀
ggggctggaAAGCTT SEQ ID NO: 124 xxxxxxx, where N is any of the four nucleotides
and xxx are the nucleotides added to the 3' and 5' ends in order to enhance the
efficiency of restriction digestion. The oligonucleotide was made double-stranded
5 with Klenow fragment, using a reverse primer xxxxxxxAAGCTTtccagcccc SEQ ID
NO: 125. After EcoR I and Hind III restriction enzyme digestion, the insert was
ligated into T7Select415-1 vector (Novagen T7Select System Manual, 1996). The
vector was packaged (Novagen T7Select System Manual, 1996) and an aliquot of the
packaging mixture was used to estimate the complexity of the library by a plaque
10 forming assay as described below. The apparent complexity of the library was 10⁸.
Packaged phage was amplified in a log phase 0.5 L culture of the BL21 E. coli strain
at 37°C for 4 hs. The cell debris was removed by centrifugation and the phage was
precipitated with 8% polyethylene glycol (M.W. 8,000) (Novagen T7Select System
Manual, 1996). Phage was extracted from the pellet with 10 mM Tris-HCl/1M NaCl/1
15 mM EDTA (pH 8.0) and stored in PBS containing 10% glycerol at -80°C (Novagen
T7Select System Manual, 1996). The amplified library used in selection experiments
displayed only truncated peptides, 1-14 amino acid residues long, immediately at the
10B protein carboxy-terminus (Table 1). The clones with full-size peptide inserts
were lost during amplification. UV-inactivated phage was prepared by irradiating 1
20 ml of phage (10¹² pfu/ml; PBS) in a well of a 6-well tissue culture plate pretreated
with BSA for 15 min under constant stirring. A germicidal lamp (15W; Sylvania
G15T8) positioned 8 cm from the sample served as a source of UV.

In Vivo Studies. Long-circulating T7 phage was selected using 180-200 g Sprague-
25 Dawley female rats pre-injected with GdCl₃ (10 mg/kg body weight) (Mizgerd, et al.,
J. Leukoc. Biol., 59:189-95. 1996) a day before. The animals were anesthetized with
Ketamine (80mg/kg) and Xylazine (4 mg/kg) and 10¹⁰ pfu (plaque-forming units) of
phage in PBS in the first or 10⁹ pfu in the following selection rounds were injected
into a tail vein. After 60 min, 6 ml of blood were collected from two animals into 600
30 units of heparin (Elkins-Sinn, Inc.) and phage in plasma were amplified as above.
Individual clones were analyzed by PCR cycle sequencing.

Persistence of phage in plasma was assessed by injecting 10^9 pfu of phage into a tail vein. At specified time points, 100 μ l blood samples were collected from a non-injected tail vein into 10 U of heparin on ice. Phage titers in blood samples on ice did not diminish over time. After centrifugation at 14,000 rpm for 5 min and 1-3 $\times 10^3$

- 5 fold dilution of the samples with LB medium, the amount of infectious phage was determined using a plaque forming assay (Novagen T7Select System Manual, 1996). Briefly, 10 μ l of diluted plasma were incubated with 250 μ l of log phase BL21 E. coli cells for 5 min, mixed with 3 ml of 0.7 % agar in LB medium and plated onto 1.5% agar in 10 cm plates. The plaques were allowed to develop overnight at room
10 temperature or over 4 hs at 37°C. The total amount of circulating phage was calculated based on a 6.4 ml plasma volume for 200 g rats (Lee and Blaufox, J. Nucl. Med., 26:72-6. 1985). C activity was inhibited by pre-injecting rats intraperitoneally with 100 μ g/kg of CVF (Calbiochem) 20 hs before the phage injection. In PC co-injection experiments, phage was injected in PBS containing 10 mM PC (Na salt).

15

- Serum preparation.** Rat serum was prepared by clotting blood on ice. Lysine-Sepharose-treated serum was prepared by passing 3 volumes of serum through 1 volume of Lysine-Sepharose (Amersham Pharmacia Biotech) equilibrated with PBS/0.68 mM CaCl₂. Complement-grade human serum from Sigma (Cat.# S-1764)
20 was reconstituted from lyophilized powder following supplier's protocol, filtered and adjusted to pH 7.4. Both rat and human sera were stored for several days on ice. CVF treatment of serum was conducted by pre-incubating serum with CVF (10.8 μ g/ml) at 37°C for 60 min.

25

Example 2- Complement Inactivation of Phage

- Given the prominent role of complement (C) in neutralizing foreign particulate material in the blood (Sakamoto, et al., Nutrition, 14:391-8. 1998), it was hypothesized that K-/R- phage were inactivated by C deposition. Pre-injecting rats with cobra venom factor (CVF) blocked *in vivo* inactivation of K-/R- phage,
30 confirming this assumption (Fig. 1 B). CVF closely mimics C3 and forms an unregulated C3/C5 convertase, which leads to depletion of C3 and C5 in the blood

and blocks further C action (Cochrane, et al., J. Immunol., 105:55-69. 1970). CVF treatment also increased recovery of the phage library from rat serum *in vitro*, from less than 1% to approximately 50%. A similar increase in phage recovery (40-60%) was obtained *in vitro* with individual K-/R- clones. Thus, the phage was inactivated

- 5 both *in vivo* and *in vitro* through C activation. Furthermore, the phage recovery *in vitro* was increased to approximately 70% by addition to serum of 10 mM Mg/EGTA; suggesting C activation through the classical pathway (Forsgren, et al., J. Lab. Clin. Med., 85:904-12. 1975).

The phage library was also inactivated by human serum. The incubation of 10^7 pfu of the phage library with 100 µl of human serum at 37°C for 30 min typically resulted in phage recovery of less than 1%. The phage recovery increased to approximately 40% and 80% after treatment with CVF and Mg/EGTA, respectively. CVF-sensitive inactivation of the phage library was also observed in mice (manuscript in preparation). Therefore, the inactivation of T7 display phage by C is a common phenomenon for different species. Human serum was different from rat serum in that it inactivated K+/R+ phage as efficiently as K-/R- phage (phage recovery < 1%).

Example 3- Inactivation of Phage Requires Natural Antibodies

20 C activation through the classical pathway is typically triggered by formation of multivalent antigen-antibody complexes (Forsgren, et al., J. Lab. Clin. Med., 85:904-12. 1975). The role of antibodies in T7 phage inactivation was determined by depleting serum of specific classes of immunoglobulins. The survival rate of phage library (10^7 pfu) in the serum depleted of IgM under routinely used conditions was 62 ± 16 % (mean ± standard deviation, n=3), as compared to 1.1 ± 0.3 % in non-treated serum. The survival of phage in IgG-depleted serum increased to 16 ± 4 % and the depletion of IgA had no effect on the phage survival rate (0.9 ± 0.3 %). The phage survival rate increased to 80-90% when more vigorous immunodepletion of IgM was conducted.

30 The addition of depleted IgM back to the IgM-depleted serum significantly restored the ability of the serum to inactivate phage library. Only 14 ± 5 % of the

phage survived under these conditions. The inactivation of phage was restored even more efficiently, decreasing the phage survival rate to $4 \pm 3\%$, with human plasma IgM from Calbiochem (Cat.# 401799). The IgM was isolated, according to the manufacturer, by gel-filtration followed by DEAE chromatography at pH 6.8. IgM
5 was eluted from DEAE-Sepharose by gradually decreasing the pH to 5.0 (Jehanli and Hough, J. Immunol. Methods, 44:199-204. 1981). Similarly prepared human myeloma IgM (Calbiochem Cat.# 401108) had no effect as the phage survival rate in reconstituted serum remained above 60%. Human plasma IgM from Sigma (Cat.# I-
8260) or Chemicon (Cat.# AG722) did not restore phage inactivation either. The
10 corresponding phage survival rates were 68 ± 13 and $74 \pm 10\%$, respectively.

The 2-D electrophoresis (O'Farrell, J. Biol. Chem., 250:4007-21. 1975) revealed that Calbiochem IgM had a wider range of isoforms and was far more acidic than Chemicon IgM. Non-reducing SDS polyacrylamide/agarose electrophoresis (Fazel, et al., Int. Immunol., 9:1149-58. 1997) showed that both preparations
15 contained predominantly pentameric IgM. Fractionation of Calbiochem IgM on Sephadryl 300 HR or Sephadryl 400 HR (Amersham Pharmacia Biotech) demonstrated that the reconstitution activity was associated with the major IgM peak. Similar results were obtained with the IgM fractionated by ammonium sulfate precipitation or ion exchange chromatography on DEAE-Sepharose (data not shown).
20 Therefore, the protein that restored phage inactivation activity to IgM-depleted serum appeared to be an acidic, predominantly pentameric form of IgM (Niles, et al., Proc. Natl. Acad. Sci., USA, 92:2884-8. 1995). The role of IgM in T7 inactivation was confirmed by comparing phage survival *in vivo* in C57BL/6J (control) and C57BL/6J Rag-1 (IgM-deficient) mice pre-injected with GdCl₃. The survival of phage in 30 min
25 after injection (10^8 - 10^9) was 20-50 times higher in C57BL/6J Rag-1 mice than in C57BL/6J mice.

The effect of IgM or IgG depletion on phage inactivation indicated that the phage inactivation was mediated by natural antibodies whose appearance in the blood does not require the presence of the corresponding antigen (Lacroix-Desmazes, et al.,
30 J. Immunol. Methods, 216:117-37. 1998). Natural antibodies are represented by IgM (Lacroix-Desmazes, et al., J. Immunol. Methods, 216:117-37. 1998) and, to a smaller

extent, by IgG (Yu, et al., J. Immunol., 157:5163-8. 1996). At least two IgM (or IgG) subunits must be simultaneously engaged in the antigen binding to initiate C activation (Cooper, Adv. Immunol., 37:151-216. 1985).

- 5 **Methods for Serum immunodepletion and reconstitution:** Human serum was depleted of IgM, IgG or IgA by passing 1 ml of serum through 2 ml of Sepharose containing immobilized goat anti-human IgM (μ -chain-specific; Sigma Cat.# A-9935), Protein G (Sigma Cat.# P-4691) and goat anti-human IgA (α -chain-specific; Sigma Cat.# A-2691), respectively. The serum was eluted from the columns with PBS
10 and collected in the total volume of 2 ml. IgM was eluted from the column by Pierce Gentle Elution Buffer, dialyzed against PBS and concentrated using Centricon-100 units. The survival of phage in immunodepleted serum was evaluated after incubating 10 μ l of phage library (10^7 pfu) with 200 μ l of serum at 37°C for 30 min. The survival of phage in reconstituted serum was assessed after incubating 10 μ l of phage library
15 (10^7 pfu) with 200 μ l of IgM-depleted serum in the presence of exogenous IgM (0.5 mg/ml). The total sample volume was adjusted to 400 μ l with PBS/0.8 mM CaCl₂.

Example 4- IgM Initiates Phage Inactivation by Binding to Displayed Peptides

To find the protein 10B determinant that bound IgM and caused C activation,
20 we analyzed the survival in human serum of a series of phage clones with truncated 10B proteins (described in the legend to Table 3). Clone 20-6 had the shortest protein 10B and a high efficiency of survival in human serum (Table 3A). Using the approach described below for identification of the plasma protein that protects K+/R+ phage against C (Fig. 2C) we found that immobilized phage 20-6 did not bind a significant
25 amount of any protein (data not shown). Nor was the resistance of clone 20-6 to C inactivation due to a particular primary structure of its 10B protein carboxy-terminus. We have found no clones with detectable resistance to C that would display peptides similar in structure to the clone 20-6 10B protein carboxy-terminus. The phage 20-6 coat protein 10B was, therefore, intrinsically resistant to C.

- 30 **Table 3. Interaction of protein 10B truncated carboxy-termini with IgM in human serum.**

Clone	Coat protein carboxy-terminus	% Phage	
		Survival	Resistant to Immunopreci- pitation*
A. 20-6	AAGAVVFQ (<u>SEQ ID NO: 61</u>)	90 ± 9	90 ± 25
B. Wild-	AAGAVVFKVE (<u>SEQ ID NO: 62</u>)	34 ± 6	126 ± 11
C. 32-77	AAGAVVFQS (<u>SEQ ID NO: 63</u>)	103 ± 8	86 ± 16
D. 32-23	AAGAVVFSQV (<u>SEQ ID NO: 64</u>)	54 ± 2	89 ± 9
E. 32-56	AAGAVVFQSE (<u>SEQ ID NO: 65</u>)	< 0.1	22 ± 3
F. 15-28	AAGAVVFQSGAAR(<u>SEQ ID NO: 66</u>)	< 0.1	3 ± 3
G. Display	AAGAVVFQSGVMLGDPNSDGA(X) ₁₋₁₄ (<u>SEQ ID NO: 67</u>)	< 1	4 ± 2

Legend to Table 3: [* mean ± standard deviation (n=3). Clones 32-23, 32-77 and 32-56 were isolated after the 1st, 3d and 4th rounds of selection for phage surviving in human serum, respectively. Clone 15-28 was selected for its ability to accumulate in rat liver (data not shown) and the selection of clone 20-6 will be described elsewhere.

- 5 Phage survival was assessed by incubating 10 µl of T7 phage (~10⁶ pfu) with 100 µl of human serum at 37°C for 30 min. Immunoprecipitation samples contained 10 µl of phage (~10⁵ pfu), 2 µl of human serum, 10 µl of 20 mM EDTA and 18 µl of PBS. EDTA was used to block C activation (Forsgren, A., et. al. *J. Lab. Clin. Med.* **85**, 904-912 (1975)). The samples were incubated at 37°C for 30 min and then treated with
- 10 200 µl of immobilized (50% agarose slurry) goat anti-human IgM (Sigma Cat.# A-9935) or anti-human IgA (Sigma Cat.# A-2691) antibodies at 4°C for 30 min. Agarose was precipitated by low-speed centrifugation and the percentage of phage resistant to immunoprecipitation was determined by titering phage in the supernatants. The recovery values for the phage treated with anti-IgM antibodies were normalized
- 15 to the corresponding values obtained with anti-IgA antibodies used as control. The absolute recovery of phage treated with anti-IgA antibodies was around 100%.

About 90% of the coat protein in wild-type phage is represented by protein 10A and only 10% by protein 10B. The wild-type protein 10A can be viewed as a protein 10B in clone 20-6 that has the carboxy-terminal Q substituted for KVE (Table 20 3B). Wild type T7 phage was quite resistant to C inactivation in human serum (Table 3B).

Clone 32-77 had one additional amino acid residue at the 10B protein carboxy-terminus relative to clone 20-6 and showed a similar efficiency of survival in human serum (Table 3C). Clone 32-23 (Table 3D) was different from clone 20-6 in that it had one additional amino acid residue (S) inserted between F and Q residues 5 and another (V) located at the carboxy-terminus. Clone 32-23 survived in serum quite well, although less efficiently than clones 20-6 and 32-77 (Table 3D). Clone 32-56 (Table 3E) had two additional amino acid residues at the carboxy-terminus of 10B protein relative to clone 20-26 and was almost completely inactivated in human serum (Table 3E). The strong inactivation of clone 32-56 was somewhat unexpected, as it 10 was isolated after 4 rounds of selection for survival in human serum. The 10B protein in clone 15-28 (Table 3F) had two additional amino acid residues (SG) from the protein 10B sequence followed by three others (AAR) of unknown origin. This clone was inactivated in human serum (Table 3F).

Thus, the appearance of phage sensitivity to C correlated with minor changes 15 in the structure of the protein 10B carboxy-terminus. In particular, the addition of as few as two amino acid residues (clone 32-56) to the protein 10B carboxy-terminus in C-resistant clone 20-6 was sufficient to render this phage C-sensitive. This is consistent with the fact that all tested K/R-clones with non-truncated coat proteins 20 were liable to C inactivation in rat serum. The survival of clones with truncated 10B proteins in serum may be explained by insufficient exposure of the C-termini of these proteins to the plasma constituents required for phage inactivation.

The minor changes in the structure of the protein 10B carboxy-terminus that rendered the phage liable to C inactivation also caused the recognition of phage by IgM. The efficiency of immunoprecipitation for C-resistant clones 20-6, 32-77 and 25 32-23 and wild-type phage in the samples treated with immobilized anti-IgM was almost as low as that in control samples treated with anti-IgA (Table 3, A-D). In contrast, the efficiency of immunoprecipitation with anti-IgM for C-sensitive clones 32-56 and 15-28, as well as for the phage library in general, was quite significant (Table 3, E-G). Therefore, the inactivation of phage by C was apparently mediated by 30 binding of IgM to the carboxy-terminal sequence of the protein 10B.

Example 5- Peptide-Specific Natural Antibodies

The basis for IgM binding to disparate 10B protein carboxy-terminal sequences in different clones was elucidated by studying the ability of phage inactivated by UV-irradiation to rescue the phage with identical or different displayed peptides from C inactivation. For example, the recovery of phage IL-14 (Table 1D and Fig. 1A, K-/R- phage) *in vivo* dramatically increased if the phage (10^9 pfu) was co-injected with an excess (10^{12} pfu) of the same phage inactivated with UV irradiation (Fig. 1B, IL-14/UV IL-14). The UV-inactivated phage IL-14 did not, however, rescue phage IL-16 (Table 1D and Fig. 1B, IL-16/UV IL-14). Other tested combinations of clones (Table 1D) gave results very similar to those shown in Fig. 1B. In addition to IL-16, UV-inactivated IL-14 did not rescue IL-1 and IL-20. UV-inactivated IL-1 rescued IL-1 but did not rescue IL-14 and IL-16. Similarly, UV-inactivated IL-21 rescued IL-21 but did not rescue IL-8 and IL-32. UV-inactivated phage also rescued live phage with identical but not with different peptides in human serum (data not shown).

Moreover, most phage with the peptides that differ from each other only in one amino acid residue will compete with each other in the inactivation assay only to a very limited extent (Table 4).

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Variable Amino Acid Residue position	UV Phage Peptide	Live Phage Peptide	Survival of Live Phage, % (mean ± SD)
-1	<u>DGAI</u> (SEQ ID NO: 68)	<u>DGAI</u>	89 ± 24
-1	<u>DGAI</u> (SEQ ID NO: 69)	<u>DGAA</u>	4 ± 2
-2	<u>DGALAS</u> (SEQ ID NO: 70)	<u>DGALAS</u>	103 ± 5
-2	<u>DGALAS</u> (SEQ ID NO: 71)	<u>DGALSS</u>	25 ± 22
-2	<u>DGADL</u> (SEQ ID NO: 72)	<u>DGADL</u>	82 ± 1

-2	<u>DGADL (SEQ ID NO: 73)</u>	<u>DGANL</u>	5 ± 6	
-3	<u>DGAGVY (SEQ ID NO: 74)</u>	<u>DGAGVY</u>	74 ± 15	
-3	<u>DGAGVY (SEQ ID NO: 75)</u>	<u>DGALVY</u>	20 ± 12	

Table 4. The specificity of the PSNA-peptide binding was evaluated by comparing the survival of phage (10^5 pfu) in rat serum (20 μ l) in the presence of an excess of UV-inactivated phage (10^{10} pfu) with the peptide that was either the same or different just 5 in one amino acid residue. The total volume of the sample was adjusted to the 240 μ l with PBS. The samples were incubated at 37°C for 30 min and the percentage of surviving live phage was determined by a plating assay.

Example 6- A Serum Factor Prevents K+/R+ Phage Inactivation

10 Serum passed through Lysine-Sepharose (that mimics peptide carboxy-terminal K residues (Deutsch and Mertz, Science, 170:1095-6. 1970) efficiently inactivated K+/R+ phage (Table 2, B and E), suggesting that K+/R+ phage was protected against C by a serum compound bound to carboxy-terminal K or R amino acid residue. The protective compound was eluted from Lysine-Sepharose by either 15 0.5 M NaCl or 2 mM EDTA, as judged from the restoration of the serum protective activity with respect to K+/R+ phage by these eluates (Table 2, C and F). As expected, Lysine-Sepharose also bound plasminogen (Deutsch and Mertz, Science, 170:1095-6. 1970). Plasminogen was eluted by ϵ -aminocaproic acid (ϵ -ACA) (Deutsch and Mertz, Science, 170:1095-6. 1970) and had no protective effect on 20 phage (data not shown).

Example 7- Identification of the Serum Protective Factor

The only major polypeptide eluted by 0.5 M NaCl or 2 mM EDTA had a molecular weight of approximately 30 kDa (Fig. 2A, lanes 1 and 2, respectively). The 25 polypeptide was identified by microsequencing (Kendrick Laboratories, Madison WI) as CRP that is normally present in rat serum at the concentration of 0.3-0.5 mg/ml (de

Beer, et al., Immunology, 45:55-70. 1982). Immunoblot analysis confirmed that the eluted protein was CRP (Fig. 2B, lane 1).

The binding of CRP to Lysine-Sepharose could be explained by a certain similarity between the lysine residues attached to Sepharose through lysine α -amino groups and the predominant CRP ligand, phosphorylcholine (PC). Like PC, the carboxy-terminal lysine contains two oppositely charged compact groups separated by a short aliphatic chain. The carboxy-terminal arginine shares this likeness. CRP is eluted from Lysine-Sepharose as a sharp peak with 1 mM PC in PBS/0.68 mM CaCl₂, which lends support to this notion (data not shown). The elution of CRP by EDTA from Lysine-Sepharose is consistent with the strict Ca²⁺-dependence of CRP-PC interaction (Volanakis and Kaplan, Proc. Soc. Exp. Biol. Med., 136:612-4. 1971).

PC reduced the protection of K+/R+ phage both *in vivo* and *in vitro*, thereby confirming the role of CRP as a K+/R+ protection agent (Fig. 1B and Table 2K, respectively). The IC₅₀ determined for K+19/16 and R+19/14 clones *in vitro* was approximately 50 μ M. Direct interaction between CRP and K+/R+ phage was shown with the phage immobilized on Affi-Gel 15. Immobilized K+/R+ phage, but not K-/R- phage, bound a substantial amount of serum protein that was eluted from the column by ϵ -ACA (Fig. 2C). SDS-PAGE showed that the major protein bound to K+ or R+ phage had the electrophoretic mobility identical to that of CRP (Fig. 2A, lanes 3 and 4). The identification of this protein as CRP was confirmed by the Western blot analysis (Fig. 2B, lanes 2 and 3). No binding of CRP to K-/R- phage or a mock column was detected. The binding of CRP to K+/R+ phage was Ca²⁺-dependent and did not take place in the presence of Mg²⁺ alone (data not shown). The presence of multiple CRP molecules on surface of K+/R+ phage could provide steric protection against C-mediated inactivation. The role of CRP in protecting K+/R+ phage against C is consistent with the lack of protection for K+/R+ phage in human serum which normally contains little CRP.

The concentration of CRP in many species, humans included, is dramatically increased as a result of an acute phase reaction (Szalai, et al., Immunol. Res., 16:127-36. 1997). The exact role of CRP *in vivo* is unclear. The functions ascribed to CRP include modulation of the immune cell behavior, participation in killing infectious

agents and clearance of cellular debris(Szalai, et al., Immunol. Res., 16:127-36. 1997). The ligands that competitively interact with the CRP PC-binding sites include phosphate monoesters (Volanakis and Kaplan, Proc. Soc. Exp. Biol. Med., 136:612-4. 1971), certain galactans (Volanakis and Narkates, J. Immunol., 126:1820-5. 1981,

- 5 Culley, et al., J. Immunol., 156:4691-6. 1996), lipoproteins and lipids (Pepys, et al., Int. Rev. Exp. Pathol., 27:83-111. 1985), immobilized laminin and fibronectin (Tseng and Mortensen, Exp. Cell. Res., 180:303-13. 1989) and cationic polymers and proteins (Dougherty, et al., Mol. Immunol., 28:1113-20. 1991, Du Clos, et al., J. Biol. Chem., 266:2167-71. 1991). Specific binding of CRP *in situ* to snRNPs (Pepys, et al.,
10 Clin. Exp. Immunol., 97:152-7. 1994) and covalent binding *in vivo* to C components C3 and C4 have been reported (Wolbink, et al., J. Immunol., 157:473-9. 1996).

Carboxy-terminal K and R residues are readily generated *in vivo* at the cell or extracellular matrix surface by trypsin-like proteases (Liotta, et al., Cancer Res., 41:4629-36. 1981). The binding of CRP to such carboxy-termini could modulate the
15 blood exposure of new protein epitopes created by extensive proteolysis. The CRP binding to proteolytic products might also play a role in the clearance of cellular debris released from dying cells (Du Clos, et al., J. Biol. Chem., 266:2167-71. 1991, Pepys, et al., Clin. Exp. Immunol., 97:152-7. 1994, Du Clos, et al., J. Immunol., 141:4266-70. 1988, Jewell, et al., Mol. Immunol., 30:701-8. 1993).

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Discussion Concerning CRP's Role in Phage Protection: CRP belongs to the pentraxin family of pentameric proteins highly conserved among different mammalian species. It was originally named because it binds the C-polysaccharide of pneumococcus and it was eventually discovered that it binds the phosphocholine moiety on the C-polysaccharide. It requires calcium for this binding. CRP can bind the phosphocholine headgroups of phospholipids such as phosphatidylcholine or sphingomyelin but not in "intact" bilayers (Richards, et al., Proc. Natl. Acad. Sci USA, 74:5672-5676. 1977). Galactosyl residues in the presence of accessible phosphocholine groups enhance CRP binding, C1q binding, and C4 activation, which
25 raises concerns about using galactosyl groups for hepatocyte targeting of liposomes (Volanakis and Narkates, J. Immunology, 126:1820-1825. 1981). Similarly, exposure

of galactosyl groups on erythrocyte membranes by desialation activates C by CRP binding(Pepys and Baltz, Advances in Immunology, 34:141-212. 1983).

Human CRP is a major acute phase reactant that is widely used for detecting inflammation- and injury-related conditions(Szalai, et al., Immunologic Research, 16:127-36. 1997). CRP is constitutively elevated in rats. The exact role of CRP *in vivo* is unclear. The functions ascribed to CRP include modulation of immune cell behavior, participation in killing infectious agents and clearance of cellular debris (Szalai, et al., Immunologic Research, 16:127-36. 1997) (Barna, et al., Cancer Research, 44:305-310. 1984). The ligands that competitively interact with the CRP PC-binding site include phosphate monoesters (Volanakis and Kaplan, Proceedings of the Society for Experimental Biology & Medicine, 136:612-4. 1971), certain galactans (Volanakis and Narkates, J. Immunology, 126:1820-1825. 1981, Culley, et al., Journal of Immunology, 156:4691-6. 1996), lipoproteins and lipids(Pepys, et al., International Review of Experimental Pathology, 27:83-111. 1985), immobilized laminin and fibronectin (Tseng and Mortensen, Experimental Cell Research, 180:303-13. 1989) and cationic polymers (polylysine, protamine, poly-arginine) and proteins (Du Clos, et al., Journal of Biological Chemistry, 266:2167-71. 1991, Dougherty, et al., Molecular Immunology, 28:1113-20. 1991) (Siegel, et al., J. Exp. Med., 142:709-721. 1975). Thus, CRP could have important effects on a number of non-viral vector systems. Specific binding of CRP *in situ* to snRNPs (Pepys, et al., Clinical & Experimental Immunology, 97:152-7. 1994) and covalent binding *in vivo* to C components C3 and C4 have been reported (Wolbink, et al., Journal of Immunology, 157:473-9. 1996).

The exact mechanism by which CRP protects Lys+/Arg+ phage against C-mediated inactivation remains to be established. Simple steric protection could be achieved just due to the presence of multiple CRP molecules on the phage surface. Bound CRP may also directly inhibit C action at the phage surface. Although CRP activates the C classical pathway (Kaplan and Volanakis, Journal of Immunology, 112:2135-47. 1974, Siegel, et al., Journal of Experimental Medicine, 140:631-47. 1974), it also inhibits the activation of the alternative and lectin pathways on the surface to which it binds. The inhibition is associated with increased C regulatory

protein H binding to C3b (Suankratay, et al., Clinical & Experimental Immunology, 113:353-359. 1998, Mold, et al., J. Immunol., 133:822-825. 1984). Perhaps, CRP's inactivation of the alternative pathway is predominant and protects the phage from C inactivation.

- 5 An important aspect of the finding that protein C-reactive protein binds to C-terminal Lys and Arg residues is a frequent occurrence of such C-termini in the blood. They are generated at the cell or extracellular matrix surface by blood trypsin-like proteases such as thrombin and plasmin (Liotta, et al., Cancer Research, 41:4629-36. 1981). The binding of CRP to such C-termini could regulate the interaction of C and immune system cells with potentially immunogenic new epitopes(Szalai, et al., Immunologic Research, 16:127-36. 1997, Suankratay, et al., Clinical & Experimental Immunology, 113:353-359. 1998). The CRP binding to proteolytic products might also play a role in the clearance of nuclear debris released from dying cells (Du Clos, et al., Journal of Biological Chemistry, 266:2167-71. 1991, Pepys, et al., Clinical &
- 10 15 Experimental Immunology, 97:152-7. 1994, Du Clos, et al., Journal of Immunology, 141:4266-70. 1988, Jewell, et al., Molecular Immunology, 30:701-8. 1993). The abundance of Lys and Arg in nuclear proteins makes proteolytic generation of C-terminal Lys and Arg aa particularly easy. It is noteworthy that the nuclear protein Sm-D that binds to CRP in solution(Jewell, et al., Molecular Immunology, 30:701-8. 1993) and is a constituent of snRNPs recognized by CRP *in situ* (Pepys, et al., Clinical & Experimental Immunology, 97:152-7. 1994) has a Lys at the C-terminus. A C-terminal Lys is also present in histone H1 that is required for CRP binding to chromatin *in vitro*(Du Clos, et al., Journal of Immunology, 141:4266-70. 1988). It should also be mentioned that all of the peptides that corresponded to nuclear protein sequences and bound to CRP *in vitro* incidentally contained a Lys or Arg at the C-terminus (Du Clos, et al., Journal of Biological Chemistry, 266:2167-71. 1991, Jewell, et al., Molecular Immunology, 30:701-8. 1993).
- 20 25

The observed selection of exclusively Lys+/Arg+ clones resulted from the combination of used selection conditions and make-up of the original library.

- 30 Lys+/Arg+ clones were initially present in the library since there was no pre-selection against truncated peptides resulting from stop-codons. The selection of Lys+/Arg+

clones was promoted as well by macrophage suppression and a long phage circulation time. Using different conditions, we were able to select Lys-/Arg- phage that persisted *in vivo* (see below). Thus, the T7 display system used herein appears to have wide applicability with respect to selecting peptide determinants recognized by blood proteins *in vivo*.

5 **Methods for Isolation and testing of CRP.** 100 ml of filtered, Sprague-Dawley rat serum (Pel-Freez Biologicals) was applied onto a 25-ml column of Lysine-Sepharose equilibrated in PBS/0.68 mM CaCl₂. The column was washed with 10 volumes of 10 PBS/0.68 mM CaCl₂ and CRP was eluted with 2 mM EDTA in PBS. CRP was dialyzed against PBS, concentrated and stored at -20°C. CRP-enriched protein fraction eluted by 0.5 M NaCl was prepared using a plasminogen isolation protocol ²¹.

10 **Methods for Affinity isolation of the serum protein protecting K+/R+ phage against Phage** was immobilized on Affi-Gel 15 (Bio-Rad) following supplier's protocol. 10¹³ pfu of phage in 3 ml of 50 mM MOPS (pH 7.5) were incubated with 4 ml of settled gel overnight at 4°C under constant mixing. The column was washed with 10 volumes of 10 mM Tris-HCl/1M NaCl/1mM EDTA/ (pH 8.0) and 10 volume of PBS. 1 ml of settled gel contained 60-120 µg of phage protein. Rat serum (3 ml) 20 was applied onto a column (4 ml) equilibrated in PBS/0.68 mM CaCl₂. The column was washed with PBS/0.68 mM CaCl₂ (8 volumes) and phage-bound protein was eluted with 2-20 mM ε-ACA in PBS/0.68 CaCl₂. 1.5 ml fractions were collected. Protein was determined by a BCA assay (Pierce). The amount of eluted protein was normalized to the amount of phage protein on the column, taking R+ phage as a 25 standard.

Example 8- Selection for Phage Persisting In Human Serum

To compare the survival strategies of phage in sera from different species, we also selected for phage that survived in human serum. 10⁹ pfu of phage library (50 µl) 30 was incubated with 1 ml of human serum (pH 7.4) at 37°C for 30 min and amplified as described above.

The phage survival rate in the 1st round of selection was less than 1%. The survival rate rose to about 10% in the 2nd round and reached approximately 35% in the 5th round of selection. The sequencing after the 1st round of selection revealed a much higher than expected (24 vs. 3%, respectively) portion of clones with tyrosine (Y) residues in displayed peptides (Y+ clones; Table 1E). Other peptides selected in the 1st round of selection showed no similarity and were not included in further analysis.

All Y+ clones showed a relatively high rate of survival in human serum. The survival rate for different Y+ clones (Table 1F) was in the range of 25 to 60%. Sequencing of the clones after the 3^d and 4th round of selection showed only a marginal increase in the percentage of Y+ clones (~30%), presumably due to a competing increase in the portion of clones with truncated 10B proteins (Table 3).

The differences in the structure of selected Y+ peptides suggested that the phage was protected against C via binding to plasma proteins rather than through assuming a specific "cryptic" conformation that is not recognized by natural antibodies. We found in our preliminary experiments with immobilized Y+ phage (clone 32-5, Table 1E) that the phage bound α_2 -macroglobulin in human serum. Consistent with this observation, the survival rate for selected Y+ clones in rat serum, that has a much lower level of α_2 -macroglobulin, was approximately 5-10-fold lower than in human serum.

Example 9 - Selecting phage T7 display clones persisting in the blood of rats not treated with gadolinium

The phage resistant to inactivation by C was selected surviving in rats with intact macrophages for 60 min. 15 individual selected clones were sequenced and all of them found to have the 10B protein truncated after Q₃₄₃⁹ as a result of a single nonsense mutation in the serine codon (TCA \Rightarrow TAA). The selected phage did not display peptides as the mutation occurred upstream of the peptide cloning site. The sequenced clones belonged to three different genotypes, as judged from the different DNA sequences at the peptide cloning site. All clones were remarkably stable in the

blood. Both with and without Gd pre-injection, the efficiency of the live phage recovery from plasma remained at the same level, in the range of 80-100%, for at least 30 min after phage injection. Approximately 4% of the injected phage could be recovered from the liver when the animals were perfused 5 min after phage injection.

- 5 The presence of phage in the liver might be due to incomplete perfusion. The spleen, lungs and kidneys showed only trace phage accumulation. The persistence of clones with different genotypes in the blood at the same level pointed to the importance of the 10B protein truncation shared by all clones for phage survival. The contribution of other, undetected mutations into phage survival could not be ruled out but, even if
10 present, was very limited. Neither in these nor in all following experiments did we detect any phage with a significant rate of survival and no obvious changes in the carboxy-terminal portion of the 10B protein. Therefore, the inactivation of the T7 phage library in rats was almost exclusively, if not entirely, driven by the structure of the 10B protein carboxy-terminus.

15

Affinity purification of an IgM species that bind specific T7 phage.

Experimental design: To elucidate more exactly the role of IgM in peptide-dependent phage inactivation, we will study if there are indeed different classes of IgM that recognize different peptides. An alternative might be the existence of some
20 other proteins that bind different classes of peptides and interact with the same class of IgM that activates complement. The specificity of an IgM species will also be confirmed.

Methods: T7 phage from the clone IL-14 (DGAKIPY) and two other clones, IL-13 (DGAVAYPPMLPVLHGSLARL (SEQ ID NO: 77)) and IL-20 (DGAYNAKTDRG
25 (SEQ ID NO: 78)), that were not protected by an excess of UV-inactivated IL-14 will be immobilized on pre-activated Affi-Gel-10 by incubating phage with Affi-Gel overnight at 4°C with end-over-end mixing. The resin will be washed with high-salt phage extraction buffer followed by Pierce Gentle Elution Buffer to eliminate a free phage contamination. Human or rat serum adjusted to the pH of 7.4 will be passed
30 through the column with immobilized phage at 4°C and the column will be washed with 5-10 volume of cold PBS containing. The serum proteins that bound to the

- immobilized phage will be eluted with Pierce Gentle Elution buffer. The eluate will be concentrated by centrifugation in Centricon 30 units and dialyzed against PBS. The resultant solution will be applied onto the affinity column containing goat antibodies (IgG) against human IgM. The column will be washed with 10 volume of PBS/0.5 M NaCl and IgM will be eluted with Pierce Gentle elution buffer. The eluted IgM will be concentrated, dialyzed against PBS and added to IgM-depleted serum to test its ability to reconstitute the inactivation by the serum of the phage clone that was used as an affinity ligand in the IgM isolation as compared to the phage clones with different peptides. Thus, the specificity of a particular IgM species will be determined.

Biotin inactivation methods

Biotinylation of T7 Phage: We have developed a novel system for selecting for phage that has been (Table 5)

15 **Table 5.** Infectivity of biotinylated T7 phage and its inactivation by neutravidin.

Concentration of Biotin-LC-NHS, mM	Neutravidin treatment	Total number of phage plaques normalized to phage dilution	Inhibition of plaque-forming activity, %
0	-	23,800	-
0.2	-	19,600	
0.2	+	72	99.63
0.5	-	15,600	
0.5	+	0	100

internalized in cells. The idea is to lightly biotinylate the phage and inactivate it with neutravidin. If the phage has been internalized then it will be inaccessible to

neutravidin and remain infectious. T7 phage can be biotinylated with only a small loss in its infectivity and this infectivity can be suppressed by neutravidin. T7 was labeled with Biotin-LC-NHS (Pierce), incubated with neutravidin (Pierce) and plated as recommended by the manufacturer (Novagen). We found that the interaction between the phage surface-conjugated biotin and free neutravidin can be used as an efficient inactivation "switch" with T7 phage display libraries. The treatment of T7 with 0.5 mM Biotin-LC-NHS resulted in very strong inhibition of phage infectivity by

- 20

neutravidin. The inhibition of phage plaque-forming activity caused by the labeling procedure itself was 30-35% (for 0.5 mM Biotin-LC-NHS).

To show the accessibility of non-internalized, endothelium-attached phage to neutravidin, we used leg muscle as a model. After intravascular injection of

- 5 biotinylated T7 phage library, the leg muscle was perfused with either PBS or PBS + neutravidin. In muscles that were perfused with neutravidin, 80% of muscle bound phage was inactivated.

Determination of whether peptides with amino termini invoke the same process.

- 10 **Experimental design:** The peptides displayed on T7 phage are cloned into the C-terminal portion of the phage coat protein 10B and, therefore, have free C-termini. In order to study the interaction with serum of the peptides that have free N-termini, we will use peptide libraries displayed on phage M13. In M13 peptide libraries, the peptides are cloned into the N-terminal portion of the phage coat proteins pIII or
- 15 pVIII.

Methods: M13 libraries that display peptides in pIII (Bio-Labs) or pVIII (courtesy of G.Smith, University of Missouri) proteins and that have complexity around 10^9 are used. Commercially available libraries from New England Bio-Labs have 7 and 12 amino acid residue long peptides. The f88-4 library from the Smith lab

20 has 15 amino acid residue long peptides in pVIII (appr. 300 copies per phage particle). The libraries are incubated in mouse serum essentially as described above for T7 phage and the surviving phage is measured by a plating assay.

All tried M13 phage display libraries are inactivated by both human and rat serum. 10^6 pfu of phage is inactivated by 100 μ l of serum during the incubation at

25 37°C for 30 min by 98-99%. A co-incubation of live phage with UV-inactivated phage (10^{10} pfu prior to inactivation) of the same type rescues the majority of live phage.

The role of displayed peptides in phage inactivation is determined by co-incubating live library with UV-inactivated vector phage or wild-type M13 phage.

- 30 The phage inactivation caused by the recognition of displayed peptides by specific natural antibodies can not be offset by the presence of an excess of wild-type phage

coat proteins. The rescue of phage with displayed peptide by an excess of inactivated wild-type phage indicates that the phage is inactivated due to the interaction of serum constituents with wild-type proteins. On the other hand, the rescue of phage with displayed peptide by an excess of inactivated phage with displayed peptides only

5 indicates that the phage is inactivated due to the interaction of serum constituents with displayed peptides.

Prolonged Circulation of Lys+/Arg+ Phage in Rats NOT Treated With Gadolinium

Although the Lys+/Arg+ phage were obtained by performing the selections in
10 rats treated with gadolinium, they had significantly prolonged circulation in rats not treated with gadolinium. Several percent of injected Lys+/Arg+phage could be recovered from rat plasma in an infectious state 5 min after injection. Almost no infectious Lys-/Arg- phage was recovered from plasma under these conditions.

Although, the actual circulation time of lys+/arg+ phage is relatively short
15 compared to sterically-stabilized liposomes, their prolonged circulation is very significant for the following reasons. One, the lys+/arg+ phage have a blood circulation time that is more than 1,000-fold above the non-selected phage given that almost all of the phage is inactivated by 5 minutes. In comparison, PEG increases the circulation times of liposomes approximately hundred-fold. One needs to appreciate
20 that the baseline for phage inactivation is different than that for liposomes which are inherently more stable in the blood than phage. Two, the fast, almost instant inactivation of most T7 clones in blood is, in fact, a very important feature of our selection system as it provides low background and short incubation times for selection of weak protein-phage interactions. Three, a relatively short circulation time
25 of CRP-protected phage appears to be due to non-specific, concentration-independent phage inactivation/clearance. A thousand-fold increase in the amount of injected Lys+ phage had no effect on the percentage of live phage in the blood. In contrast, a thousand-fold increase in the amount of injected Lys- (not recognized by CRP) phage resulted in a dramatic increase in the survival of this phage in the blood. The survival
30 of Lys- phage under these conditions was quantitatively very similar to that of Lys+ phage. Further increase in the amount of Lys- injected phage had no effect on the

survival percentage. These results indicate that the circulation time of our selected phage is quite close to the maximum that can be obtained with this system. Artificial delivery vectors bearing the lys+/arg+ peptides may not be subject to this non-specific inactivation/clearance.

5

Selection of Phage Clones Resistant to Human Complement Inactivation

The binding and precipitating properties of CRPs from different species show certain. Further studies are necessary to extend our initial findings in the rat serum to human serum. "Lysine-/arginine-" phage are 100% inactivated by "normal" human serum containing just trace amounts of CRP. The extent of inactivation by human serum is comparable to that by rat serum. Therefore, the inactivation of T7 phage by serum is a universal phenomenon, not confined to a particular species. Since human serum is naturally low in CRP (< 10 µg/ml except during an acute phase response), we reasoned that selections in human serum would yield serum resistant "lys-/arg-" clones containing novel peptide sequences not obtained from the rat studies.

Selection of peptides that confer resistance on T7 phage against human complement was carried out using pooled commercial "complement grade" serum from Sigma (Cat. # S1764) and a new T7 peptide library containing 1-13 amino acid long linear peptides. The selection was performed under relatively "mild" selection pressure, using a relatively large phage/serum ratio. Lyophilized serum was reconstituted with water and filtered through a 0.22 µm filter prior to use. 10^9 pfu of the T7 library in 200 µl of PBS were incubated with 1 ml of serum for 30 min and complement-resistant phage was amplified in a 0.5 L log culture of BL 21 E. coli. Amplified phage was isolated and the procedure was repeated 2 more times using the same conditions. 30 individual clones were isolated from the selected phage population, sequenced and grouped according to certain consensus residues (Table 5).

An obvious trait shared by many selected peptides was the high frequency occurrence of Ser and Thr residues near the C-terminus. 6 clones particularly stood out in that they had double or triple cysteins at the C-terminus. All other clones are presented in Table 5 according to the position of Ser/Thr residues in the corresponding peptides.

Table 5. Peptide sequences of the 30 individual clones that were obtained after selection for resistance to inactivation by human serum. The clones were divided into A-F groups according to the following criteria A. Peptides with double or triple Ser residues at the C-terminus; B. Peptides with single Ser residues at the C-terminus; C. Peptides with a Ser residue at the -2 position; D. Peptides with a Ser residue at the -3 position; E. Peptides containing Ser or Thr residues at any other but C-terminal, -2 or -3 positions ; F. Peptides that do not contain Ser and Thr residues. Ser and Thr residues are shown in bold. The linker portion of 10B protein upstream of random peptides (10B) is underlined

*Clones 24-1 and 24-24 are genetically different (i.e., non-coding sequences are different).

Table 5. Clones selected for resistance against inactivation by human serum

A. Multiple COOH Ser 24-1 <u>DGALSS*</u> (SEQ ID NO: 79) 24-10 <u>DGAHSSS</u> (SEQ ID NO: 80) 24-11 <u>DGASNLLSS</u> (SEQ ID NO: 81) 24-16 <u>DGAARNTLSS</u> (SEQ ID NO: 82) 24-21 <u>DGAAISSLGFINQSS</u> (SEQ ID NO: 83) 24-24 <u>DGALSS*</u> (SEQ ID NO: 84)	B. Single COOH Ser 24-15 <u>DGALAS</u> (SEQ ID NO: 85) 24-22 <u>DGAWS</u> (SEQ ID NO: 86)	C. Ser at -2 Position 24-3 <u>DGANSP</u> (SEQ ID NO: 87) 24-8 <u>DGASSV</u> (SEQ ID NO: 88) 24-12 <u>DGASDRGNEEMSF</u> (SEQ ID NO: 89)
D. Ser at -3 Position 24-6 <u>DGAMSPL</u> (SEQ ID NO: 90) 24-14 <u>DGAVPSVSSPSIG</u> (SEQ ID NO: 91) 24-23 <u>DGASGPSVG</u> (SEQ ID NO: 92) 24-27 <u>DGATTSLG</u> (SEQ ID NO: 93) 24-28 <u>DGSQM</u> (SEQ ID NO: 94)	E. Ser or Thr/Not COOH 24-4 <u>DGAPSLSVGG</u> (SEQ ID NO: 95) 24-5 <u>DGATTVDNM</u> (SEQ ID NO: 96) 24-7 <u>DGANLVSGTRLD</u> (SEQ ID NO: 97) 24-9 <u>DGATG</u> (SEQ ID NO: 98) 24-17 <u>DGATTQTAY</u> (SEQ ID NO: 99) 24-18 <u>DGASNPL</u> (SEQ ID NO: 100)	F. No Ser or Thr 24-2 <u>DGAVPL</u> (SEQ ID NO: 104) 24-13 <u>DGARA</u> (SEQ ID NO: 105) 24-20 <u>DGAMVG</u> (SEQ ID NO: 106) 24-26 <u>DGAVRRG</u> (SEQ ID NO: 107) 24-29 <u>DGAALVL</u> (SEQ ID NO: 108)

	24-19 <u>DGAATRGR</u> (<u>SEQ ID NO: 101</u>) 24-25 <u>DGASKKTVLAMNPR</u> (<u>SEQ ID NO: 102</u>) 24-30 <u>DGATHGSEVA</u> (<u>SEQ ID NO: 103</u>)		
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To evaluate the efficiency of selection, all selected clones were amplified and tested individually for survival in human serum. Individual clones were tested for survival in human serum by incubating 10 µl of T7 phage with 300 µl of reconstituted serum diluted with 190 µl of PBS. The incubation was carried out at 37°C for 30 min and the efficiency of phage survival was estimated by plating as described earlier. Percentage of T7 phage recovered (X axis) equals (# of recovered phage /# of injected phage) X 100. 10⁷ PFU's were used. We found that all selected clones with double Ser residues at the C-terminus showed a significant resistance to complement inactivation (~15 to ~55 percent survival). Furthermore, the presence of double Ser right at the C-terminus appears to be critical for phage survival. Thus, clone 24-8 that displayed peptide SSV showed a low resistance to complement inactivation (< 1 % survival) despite the presence of Ser residues at -2 and -3 positions. In addition, it is not clear whether the presence of a single Ser residue at the C-terminus is sufficient to protect phage against complement. Out of two selected clones with a single Ser residue at the C-terminus, one was quite resistant to serum inactivation (24/15) while the other (24/22) was totally inactivated. It is necessary to examine further clones to determine the role of other residues on the ability of a single C-terminal Ser to confer resistance to human serum. A number of selected clones that showed resistance to complement inactivation did not have peptides with Ser residues at the C-terminus (Table 6). Most interestingly, this suggests that selected peptides protect phage against complement inactivation via different mechanisms.

Table 6. Selected peptides that confer on T7 resistance to complement inactivation and do not have C-terminal Ser residues. Peptides in columns A and B are grouped based on a certain amount of similarity in the primary structure of C-termini.

A. 24-6 <u>DGAMSPL</u> (SEQ ID NO: 109) 24-18 <u>DGASNPL</u> (SEQ ID NO: 110) 24-29 <u>DGAALVL</u> (SEQ ID NO: 111)	B. 24-19 <u>DGAATRGR</u> (SEQ ID NO: 112) 24-26 <u>DGAVRRG</u> (SEQ ID NO: 113)	C. 24-5 <u>DGATTVDNM</u> (SEQ ID NO: 114) 24-17 <u>DGATTQTAY</u> (SEQ ID NO: 115) 24-25 <u>DGASKKTVLAMNPR</u> (SEQ ID NO: 116) 24-27 <u>DGATTSLG</u> (SEQ ID NO: 117) 24-28 <u>DGSQM</u> (SEQ ID NO: 118)
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The exact mechanisms of phage protection by selected peptides are currently being studied. There are two possible mechanisms: one, the peptide binds a serum protein that prevents C inactivation, or the two Ser residues act like PEG and reduce all serum protein interactions. Our proposed methods of using phage affinity columns (see Exp Protocol section) will distinguish between these two hypotheses. If it should be the first mechanism, then the exact serum proteins that bind the phage and prevent C inactivation will be identified (just as we did with the identification of CRP for rat serum).

In addition, it appears that the C terminus carboxyl group plays an important role since acidification of the serum abrogates the protection of the above selected clones. Further selections in human serum with adjusted pH's are in progress.

Inactivation of phage display library by pup rat serum and the serum from gnotobiotic rats.

Serum from newborn rats did not inactivate phage to a significant extent. Only 7.7+-5.6% of the input phage was inactivated as compared to 96.3+-0.3 % in the serum from adult rats. The inactivation of phage by the serum of new-born rats could be induced by adding to the samples 100 µg of IgM (purified as described above). The efficiency of phage inactivation increased under these conditions to 70.5+-4%. This suggests that the peptide-specific IgM develops in the neonatal period. Serum from gnotobiotic rats *in vitro* and *in vivo* inactivated phage as effectively as the serum from control animals indicating that the appearance of "active IgM" is not induced by environmental agents.

Liver targeting and blood persistence of T7 phage with truncated proteins in rats

We have identified a number of spontaneous mutations that occur around the site corresponding to the translation shift point from 10A to 10B protein reading frame. Most of these phage clones were selected due to their resistance to serum inactivation. Interestingly, we discovered that their targeting behavior *in vivo* showed a wide spectrum of behavior (FIG. 1).

Both clones 20/6 (FQ*) and 32/77 (FQS*) are relatively stable in the blood but differ substantially in their liver targeting (Fig. 1). The one additional C-terminus serine in 32/77 results in over 20% of the injected phage ending up in the liver.

Clones 32/33 (FSQV (SEQ ID NO: 119)) and #112 (FQSGVMLGDPN* (SEQ ID NO: 120)) also target to the liver but are not as stable in blood. Clones 114 and T7 vector, shown for control purposes, are not stable in blood and can not be detected in liver liver. The overall impression from these experiments is that liver targeting is associated with the exposure of peptides on the phage surface, regardless of the peptide primary structure.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in cell biology, chemistry, molecular biology, biochemistry or related fields are intended to be within the scope of the following claims.



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